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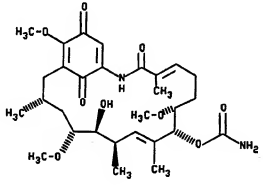

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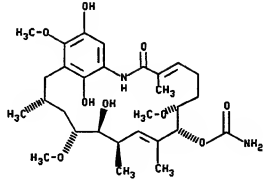

(II)

(57) Abstract

A process for the fermentation and isolation of compounds (I) and (II), members of the ansamycin benzoquinone antibiotics family, having formulae (I) and (II), as well as the chemical synthesis of compound (II) from compound (I) are disclosed in the present invention. The compounds of the present invention are useful against proliferative disorders including, but not limited to, cancer in mammals, especially humans. The compounds of the present invention are also expected to be useful against certain microorganisms, and have utility as immunosuppressive agents against autoimmune diseases.

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PROCESS AND USES FOR 4,5-DIHYDROGELDANAMYCIN AND ITS HYDROQUINONE

Background of the Invention

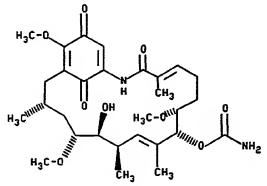
This invention is concerned with a new process for the preparation of 4,5-dihydrogeldanamycin and its hydroquinone by fermenting the microorganism Streptomyces hygroscopicus, Pfizer culture collection number FD 29068, a derivative by subculture from NRRL 3602, now deposited as ATCC 55256, using standard fermentation methods and conditions, followed by isolating the compounds of this invention using standard separation methods. The hydroquinone can also be chemically synthesized from 4,5-dihydrogeldanamycin.

Both 4,5-dihydrogeldanamycin and its hydroquinone are chemical compounds belonging to the ansamycin benzoquinone family of antibiotics. 4,5-Dihydrogeldanamycin and its hydroquinone are considered to be derivatives of geldanamycin, a well-known member of the ansamycin benzoquinone family. Geldanamycin itself is obtained by fermenting the microorganism Streptomyces hygroscopicus, NRRL 3602, and separating it out from the fermentation broth. Geldanamycin is known to be useful against certain microorganisms, primarily yeast and fungi. Geldanamycin's preparation and utility are disclosed in U.S. Patent 3,595,955. 4,5-Dihydrogeldanamycin has previously been synthesized by catalytically hydrogenating geldanamycin. Reference to the semisynthesis of 4,5-dihydrogeldanamycin is found in Progress in the Chemistry of Organic Natural Products, Chemistry of the Ansamycin Antibiotics, 33, 1976, p. 278. As of this date, no utility for 4,5-dihydrogeldanamycin has been disclosed in the art.

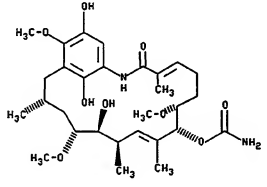
Semisynthetic derivatives of geldanamycin and their use as antitumor agents are described in Derwent abstracts 82-98300E, 81-70796D, 80-72388C and 80-62760C.

Summary of the Invention

This invention provides a process for preparation of 4,5-dihydrogeldanamycin which has the chemical formula I, and the hydroquinone of 4,5-dihydrogeldanamycin which has the chemical formula II, below.



(I)



(II)

The process comprises the submerged aerobic propagation in aqueous nutrient media of the microorganism Streptomyces hygroscopicus, ATCC 55256, followed by isolation of the compounds of formulae I and II. The inventors have discovered that 4,5-dihydrogeldanamycin and its hydroquinone can be obtained by fermenting Streptomyces hygroscopicus, ATCC 55256, a microorganism not previously known to produce the compounds of this invention. 4,5-Dihydrogeldanamycin is also shown to have valuable utility in treating proliferative disorders, especially cancer, in mammals and, more specifically, in humans. 4,5-Dihydrogeldanamycin is also expected to be useful in treating certain gram-positive and -negative bacterial

infections; useful as a virucidal agent and as a herbicide and possess antifungal and antiprotozoal properties. Because the compound also possesses immunosuppressive properties, it is expected to be useful in treating a variety of autoimmune diseases including but not limited to rheumatoid arthritis and graft versus host diseases.

The hydroquinone of 4,5-dihydrogeldanamycin is a novel compound that can be isolated from a natural source or chemically synthesized. The hydroquinone can be isolated from the fermentation broth of Streptomyces hygroscopicus in essentially the same manner that geldanamycin and 4,5-dihydrogeldanamycin is isolated. The hydroquinone can also be chemically synthesized by reducing 4,5-dihydrogeldanamycin with a chemical reducing agent. The hydroquinone of 4,5-dihydrogeldanamycin is expected to be useful against all of the same diseases as listed above for 4,5-dihydrogeldanamycin. The instant invention provides a new process for the preparation of 4,5-dihydrogeldanamycin and 4,5-dihydrogeldanamycin hydroquinone from a natural source, namely Streptomyces hygroscopicus, ATCC 55256, and provides new uses for the compounds of this invention. Also, the chemical synthesis of the hydroquinone from 4,5-dihydrogeldanamycin is provided.

Detailed Description of the Invention

Streptomyces hygroscopicus, NRRL 3602, also referred to as Pfizer culture collection number FD 29068, has been deposited under the terms of the Budapest Treaty in the American Type Culture Collection, Rockville, Maryland, a recognized depository affording permanence of the deposits and ready accessibility thereto by the public if a patent is granted on this application. It has been given the designation Streptomyces hygroscopicus ATCC 55256. The deposit is available during pendency of this application to one determined by the Commissioner of the United States Patent and Trademark Office to be entitled thereto under 37 CFR 1.14 and 35 USC 122, and in accordance with foreign patent laws in countries wherein counterparts of this application, or its progeny, are filed. All restrictions on the availability to the public of the microorganism deposited will be irrevocably removed on June 2, 1993 or upon granting of the patent, whichever is earlier.

Compounds (I) and (II) are natural products which can be isolated from the fermentation broth of Streptomyces hygroscopicus, ATCC 55256. The method of propagating Streptomyces hygroscopicus, ATCC 55256, to obtain 4,5-

dihydrogeldanamycin and its hydroquinone is the same as for propagating it to obtain geldanamycin. The method of propagation is standard in the art and can be found in U.S. Patent 3,595,955, the teachings of which are incorporated herein by reference. The Streptomyces hygroscopicus, ATCC 55256, culture can be grown at 24° to 36°C under submerged conditions with agitation and aeration on media consisting of carbohydrate sources such as sugars, starches, glycerol; organic nitrogen sources such as soybean meal, casamino acids, yeast extract; growth substance such as grain solubles, fish meal, cotton seed meal; mineral salts containing trace elements such as iron, cobalt, copper, zinc, etc. Inoculum is prepared by scraping vegetative cells from slants inoculated with the ATCC 55256 culture. A suitable solid medium for initial growth on slants is ATCC no. 172, the components of which are listed below.

ATCC #172	Grams/liter
Glucose	10
Soluble Starch	20
Yeast Extract	5
*NZ Amine A	5
Calcium Carbonate	1
Agar	20

*Added distilled water to 1000 ml and adjusted the pH to 7.0 with KOH.

*NZ Amine A is a registered trademark of Kraft, Inc., Product of Quest International (Sheffield Products).

Cultivating Streptomyces hygroscopicus, ATCC 55256, and isolating the compounds of formulae I and II is conducted in a similar manner to those employed in previous fermentations yielding geldanamycin. See, for example, U.S. Patent Number, 3,595,955. Cultivation preferably takes place in aqueous nutrient media under submerged aerobic conditions with agitation at a temperature of 24° to 36°C. Nutrient media useful for cultivation include a source of assimilable carbon such as sugars, starches and glycerol; a source of organic nitrogen such as soybean meal, casamino acids and yeast extracts. A source of growth substances such as grain solubles, fish meal and cotton seed meal as well as mineral salts such as sodium chloride and trace elements such as iron, cobalt, copper and zinc. Buffering agents such as calcium carbonate and phosphates are used as well. If excessive foaming is encountered during fermentation, antifoam agents such as vegetable oils or silicones may be added to the fermentation medium. Aeration of the medium in

tanks for submerged growth is preferably maintained at the rate of about 1/2 to 2 volumes of sterile free air per volume of fermentation broth per minute forced into the broth through a sparger. Agitation may be maintained by means of agitators generally familiar to those skilled in the fermentation art. The rate of agitation depends on the type of agitator employed. A shaker flask is usually run at 150 to 200 cycles per minute whereas a fermentor is usually run at 300 to 1700 revolutions per minute. Aseptic conditions must, of course, be maintained through the transfer of the organism and throughout its growth.

Inoculum for the preparation of the antibiotics according to this invention may be obtained by employing growth from a slant of the culture or Roux bottles inoculated with the culture. A solid medium suitable for initial growth of the organism on slants and in Roux bottles is ATCC medium no. 172. The growth may be used to inoculate either shaker flasks or inoculum tanks or the inoculum tanks may be seeded from the shaker flasks. Growth in shaker flasks will generally have reached its maximum in 4 to 5 days whereas inoculum in submerged inoculum tanks will usually be in the most favorable period in 2 to 3 days.

Nigercin and elaiophilin, which are active against gram-positive and negative microorganisms, are coproduced in the fermentation broth and are good indicators of growth associated with the production of 4,5-dihydrogeldanamycin. Therefore, the bioactivity of the fermentation broth can be monitored by biological assay of the broth employing a sensitive strain of Staphylococcus aureus ATCC 6538P or Bacillus subtilis ATCC 6633. Standard plate assay techniques are employed in which the zone of inhibition surrounding a filter paper disc saturated with the broth is used as a measure of antibiotic potency. Also, thin-layer chromatography employing silica gel is a useful tool for detecting the antibiotics produced in the fermentation media and analyzing the composition of the crude and purified materials extracted from the fermentation broths. The chromatograms are developed with ethyl acetate and the antibiotic compounds are visualized by spraying with vanillin reagent and heating the TLC plate at 80°C. The developed plate can also be overlayed with agar seeded with either S. aureus or B. subtilis and incubated at 37°C for 16 hours to visualize the antibiotics.

Compounds of formulae I and II produced by fermentation of Streptomyces hygroscopicus, ATCC 55256, may be separated and recovered by conventional methods, e.g., extracting the whole, unfiltered fermentation broth with an organic

solvent such as chloroform, ethyl acetate, methyl isobutyl ketone or butanol at the naturally prevailing pH. Alternatively, the mycelium can be separated after growth has been completed and the mycelium extracted with an organic solvent. The solvent extract can then be concentrated to a thin syrup and the pure antibiotic obtained by chromatography.

A typical method of separation and recovery of the compounds of formulae I and II is as follows. The whole broth from fermentation of Streptomyces hygroscopicus, ATCC 55256, is extracted with methyl isobutyl ketone. The solvent is evaporated to yield a thin syrup. The syrup is dissolved in methylene chloride, loaded onto a silica gel column and eluted with a gradient of neat methylene chloride to neat ethyl acetate. The eluates are examined by thin-layer chromatography. Fractions containing compound I are combined and evaporated to dryness. Fractions containing compound II are combined and evaporated to dryness. The products may be further purified by crystallization or by column chromatography if desired.

The compounds of formulae I and II are expected to be useful against certain genera of fungal plant pathogens, gram-positive and negative bacteria and certain parasitic microorganisms. 4,5-Dihydrogeldanamycin and its hydroquinone can be tested for use against the above mentioned microorganisms using the method disclosed in U.S. Patent 3,595,955.

The compound of formula I also inhibits the growth of certain human carcinoma cells. The in vitro activity of 4,5-dihydrogeldanamycin was determined according to the method contained in M.C. Alley et al. Cancer Research 48, 589-601, Feb. 1, 1988, and using SKBR3 and MCF7 cell lines. Therefore, 4,5-dihydrogeldanamycin is particularly valuable in treating cancer, and especially breast, ovarian and gastric cancer in humans. The compound of formula II is expected to be useful for the same purpose.

4,5-Dihydrogeldanamycin also has potent immunosuppressive effects as determined by methods well known to those skilled in the art. This activity can be conveniently determined by assessing the inhibition of T-cell proliferation stimulated by IL-2 and phorbol 12-myristate 13-acetate (PMA) as measured by a reduction in uptake of tritiated thymidine relative to non-drug treated controls. Compound II is also expected to have immunosuppressive effects.

When the compounds of formulae I or II are to be used as an antiproliferative agent, such as an anticancer agent, it can be administered to a mammalian subject either alone or, preferably, in combination with pharmaceutically-acceptable carriers or diluents in a pharmaceutical composition according to standard pharmaceutical practice. The compounds can be administered orally or parenterally. Parenteral administration includes intravenous, intramuscular, intraperitoneal, subcutaneous and topical administration.

For oral use of a compound of formula I or II of this invention, the compound can be administered, for example, in the form of tablets or capsules, or as an aqueous solution or suspension. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch, and lubricating agents, such as magnesium stearate, are commonly added. For oral administration in capsule form, useful diluents are lactose and dried corn starch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added. For intramuscular, intraperitoneal, subcutaneous and intravenous use, sterile solutions of the active ingredient are usually prepared, and the pH of the solutions should be suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

In a pharmaceutical composition comprising the compound of formula I or II the weight ratio of carrier to active ingredient will normally be in the range from 1:10 to 10:1. However, in any given case, the ratio chosen will depend on such factors as the solubility of the active component, the dosage contemplated and the precise route of administration.

When the compound of formula I or II is used in a human subject, the daily dosage will normally be determined by the prescribing physician. Moreover, the dosage will vary according to the age, weight and response of the individual patient, as well as the severity of the patient's symptoms and the potency of the particular compound being administered. However, an effective dose in most instances will be 0.01 to 0.5g as needed (e.g., every four to six hours). For chronic administration, in most instances, an effective dose will be from 0.01 to 1.0 g per day, and preferably 20 to 250 mg per day, in single or divided doses. On the other hand, it may be necessary to use dosages outside these limits in some cases.

The following examples are being provided solely for the purpose of further illustration.

EXAMPLE 1

1. Preparation of Inoculum

A solid medium suitable for initial growth on slants and Roux bottles is ATCC medium No. 172. To 300 ml shaker flasks, 100 ml of medium was distributed, then the shaker flasks were sterilized at 120°C and 15 p.s.i. for 30 minutes. After cooling, the medium was inoculated with a vegetative cell suspension from the Streptomyces hygroscopicus, ATCC 55256, culture grown on ATCC no. 172 medium agar. The flasks were shaken at 28°C on a shaker having a displacement of 1-1/2 to 2-1/2 inches and 150 to 200 cycles per minute (CPM) for 3 to 5 days.

Shaker flasks are prepared using one of the following media:

JDYTT	Grams/liter
Cerelose	10
Corn Starch	5
Corn Steep Liquor	5
NZ Amine YTT	5
Cobalt Chloride	0.002
Calcium Carbonate	3

pH to 6.9~7.1

C¹	Grams/liter
Cerelose	10
Soy Flour	10
Corn Fermentation Products	5
Corn Starch	10
Sodium Chloride	5
Cobalt Chloride	0.002
Calcium Carbonate	1

pH to 7.0~7.2

2. Fermentation and isolation of 4,5-Dihydrogel-danamycin

One shaker flask is used to inoculate a five-liter fermentation vessel containing three liters of one of the following media:

HERB-F	Grams/liter
Cerelose	25
Ammonium Sulfate	5
Soybean Flour	10
Yeast Extract	2.5
Potassium Chloride	4
Meat Extract	1
Cobalt Chloride	0.002
Calcium Carbonate	3
	pH to 7.1~7.3

HERB-F2	Grams/liter
Cerelose	10
Corn Starch	40
Cotton Seed Meal	4
Cobalt Chloride	0.002
Calcium carbonate	6
Brewers Yeast	2
Sodium Chloride	2
Magnesium Sulfate•$7H_2O$	0.5
Ammonium Nitrate	2
	pH to 6.9~7.2

MACB-M	Grams/liter
Glycerol	10
Yeast Extract	10
Sodium Nitrate	2
Cobalt Chloride	0.002
Magnesium Sulfate•$7H_2O$	0.50
Potassium Dibasic Phosphate	1
Potassium Chloride	0.5
Ferrous Sulfate	0.01
	pH to 6.9~7.2

To each vessel was added 1 ml of P2000 (silicone) as an antifoaming agent, then the vessels were sealed and sterilized at 120°C and 15 p.s.i. for one hour. Then, the pots were inoculated with one (ca. 3% inoculum) flask, fermented for 72-120 hours at 28°C and stirred at 1700 revolutions per minute with an air rate of one volume of air per volume of liquid per minute.

The bioactivity of the broth and subsequent recovery streams was followed by using a sensitive strain of Bacillus subtilis ATCC 6633 or Staphylococcus aureus

ATCC 6538P. The components in the broth and recovery streams were visualized by using silica gel plates in the following system: neat ethyl acetate. The developed plates were sprayed with vanillin reagent (3 g vanillin dissolved in 75 ml of ethanol and diluted to 100 ml with 85% phosphoric acid) then heated to 80°C. The antibiotics appear as a deep blue/purple coloration. The developed thin-layer chromatography (TLC) plate was also visualized by viewing in a dark box with 254 μm light. When fermentation was completed the fermenters were stopped. The whole broth was extracted with 1/3 volume of methyl isobutyl ketone at broth pH, separated on a DeLaval separator and the solvent phase concentrated in vacuo to an oil. The oil was subjected to a 3 tube countercurrent using hexane/acetonitrile 10:1. The lower phase (CH_3CN) containing 4,5-dihydrogeldanamycin and its hydroquinone was separated, combined and concentrated in vacuo. The residue was redissolved in methylene chloride, treated with Darco G60 (activated carbon), filtered and concentrated. The concentrate was added to a Waters Prep 500 silica gel column in methylene chloride, then subjected to a gradient of neat methylene chloride to neat ethyl acetate. Fractions enriched with 4,5-dihydrogeldanamycin, R_f=2.5-3 in 9:1 $CHCl_3$/Acetone, were combined and subjected to repeated chromatographies until pure 4,5-dihydrogeldanamycin was isolated. 4,5-Dihydrogeldanamycin was crystallized from hot isopropyl ether and dried in a vacuum oven at 50°C overnight. m.p. 221-222°C.

Fractions enriched with the hydroquinone R_f=1-1.5 in 9:1 $CHCl_3$/Acetone, of formula II were combined and subjected to repeated chromatographies until pure hydroquinone was isolated.

EXAMPLE 2

Chemical Synthesis of 4,5-dihydrogeldanamycin hydroquinone

The reaction was commenced by mixing 10 grams of sodium hydrosulfite dissolved in 100 ml of water with 100 ml of ethylacetate, then 200 mg of 4,5-dihydrogeldanamycin was added to the above solution. The reaction was followed by tlc using Analtech silica gel plates and neat ethyl acetate as the system. The reaction was observed under UV light (254 μm) and by vanillin spray. The reaction was almost complete after 20 minutes.

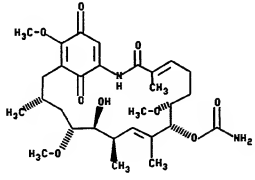
The aqueous layer was extracted twice with ethyl acetate. Then, the solvent layer was back extracted with pH 7.0 phosphate buffer. The solvent was dried over anhydrous Na_2SO_4 and concentrated to a gum.

The residue was taken up in isopropyl ether (IPE), heated to a boil and then stirred for 3 hours. The isopropyl ether solution was filtered and the solid dried on the filter. The dried 4,5-dihydrogeldanamycin was kept under house vacuum at 50°C.

Claims

What is claimed is:

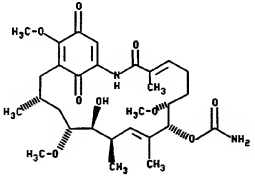
1. A process for preparing a compound of the formula



(I)

which comprises the steps of (a) propagating, in an aqueous nutrient medium, under submerged aerobic condition, the microorganism Streptomyces hygroscopicus, ATCC 55256, wherein the nutrient medium comprises a carbohydrate source, an organic nitrogen source, a growth substance and mineral salts containing trace elements; and (b) isolating the compound of formula (I).

2. A method of treating a proliferative disorder in a mammalian subject which comprises administering to said mammalian subject a proliferative disorder treating amount of the compound of formula I



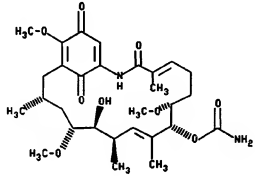
(I)

3. A method according to claim 2 wherein said compound is administered orally.

4. A method according to claim 2 wherein said compound is administered parenterally.

5. A method according to claim 2 wherein the proliferative disorder is human breast, ovarian or gastric cancer.

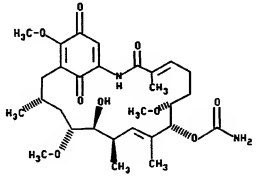
6. A method of treating an autoimmune disease in a mammalian subject which comprises administering to said mammalian subject an autoimmune disease treating amount of the compound of formula I



(I)

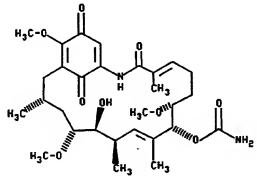
7. A method according to claim 6 wherein the autoimmune disease is rheumatoid arthritis or graft versus host disease.

8. A method of treating a mammalian subject suffering from a bacterial, viral, fungal or protozoal infection which comprises administering to said mammalian subject a bacterial, viral, fungal or protozoal infection treating amount of the compound of formula I



(I)

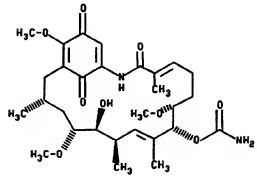
9. A pharmaceutical composition useful in the treatment of a proliferative disorder in a mammalian subject which comprises a pharmaceutically-acceptable carrier or diluent and a proliferative disorder treating amount of the compound of formula I



(I)

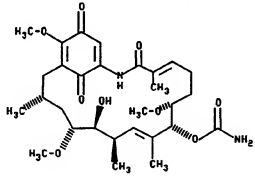
10. A pharmaceutical composition useful in the treatment of an autoimmune disease in a mammalian subject which comprises a pharmaceutically-acceptable

carrier or diluent and an autoimmune disease treating amount of the compound of formula I



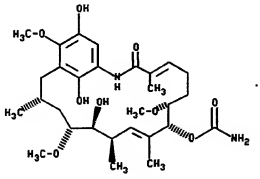
(I)

11. A pharmaceutical composition useful in the treatment of a bacterial, viral, fungal or protozoal infection in a mammalian subject which comprises a pharmaceutically-acceptable carrier or diluent and a bacterial, viral, fungal or protozoal infection treating amount of the compound of formula I



(I)

12. A compound of the formula



(II)

13. A process for preparing the compound of formula (II) according to claim 12 which comprises the steps of (a) propagating, in an aqueous nutrient medium, under submerged aerobic condition, the microorganism Streptomyces hygroscopicus, ATCC 55256, wherein the nutrient medium comprises a carbohydrate source, an organic nitrogen source, a growth substance and mineral salts containing trace elements; and (b) isolating the compound of formula (II).

14. A method of treating a proliferative disorder in a mammalian subject which comprises administering to said mammalian subject a proliferative disorder treating amount of the compound according to claim 12.

15. A method according to claim 14 wherein said compound is administered parenterally.

16. A method according to claim 14 wherein said compound is administered orally.

17. A method according to claim 14 wherein the proliferative disorder is human breast, ovarian or gastric cancer.

18. A method of treating an autoimmune disease in a mammalian subject which comprises administering to said mammalian subject an autoimmune disease treating amount of the compound according to claim 12.

19. A method according to claim 18 wherein the auto-immune disease is rheumatoid arthritis or graft versus host disease.

20. A method of treating a mammalian subject suffering from a bacterial, viral, fungal or protozoal infection which comprises administering to said mammalian

subject a bacterial, viral, fungal or protozoal infection treating amount of the compound according to claim 12.

21. A pharmaceutical composition useful in the treatment of a proliferative disorder in a mammalian subject which comprises a pharmaceutically-acceptable carrier or diluent and a proliferative disorder treating amount of the compound according to claim 12.

22. A pharmaceutical composition useful in the treatment of an autoimmune disease in a mammalian subject which comprises a pharmaceutically-acceptable carrier or diluent and an autoimmune disease treating amount of the compound according to claim 12.

23. A pharmaceutical composition useful in the treatment of a bacterial, viral, fungal or protozoal infection in a mammalian subject which comprises a pharmaceutically-acceptable carrier or diluent and a bacterial, viral, fungal or protozoal infection treating amount of the compound according to claim 12.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/10189

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 P 17/10,C 12 P 1/06,A 61 K 31/395,C 07 D 225/06, //(C 12 P 1/06,C 12 R 1:55)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 P,A 61 K,C 07 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 3 595 955 (C. DE BOER et al.) 27 July 1971 (27.07.71), claims. --	1,11-13,23
A	Chemical Abstracts, vol. 74, no. 13, issued 1971, March 29 (Columbus, Ohio, U.S.A.), K.L. RINEHART et al. "Geldanamycin. I. Structure assignment", page 301, right column, the abstract-no. 64 194b, J. Amer. Chem. Soc. 1970, 92(26), 7591-3. --	1,12
A	Patent Abstracts of Japan, unexamined applications, C field, vol. 4, no. 173, November 29, 1980, The Patent Office Japanese Government, page 67 C 32,	1,12

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 February 1993	15 MAR 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	WOLF e.h.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	No. 55-111 419 (KAKEN KAGAKU), Derwent abstract 80-723 88C.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/ 10189

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 2-8,14-20
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv)
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/US 92/10189 SAE 67918

In diesem Anhang sind die Mitglieder der Patentfamilien der in obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
US A 3595955	27-07-71	keine - none - rien	